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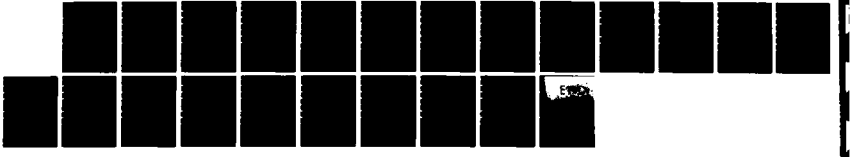

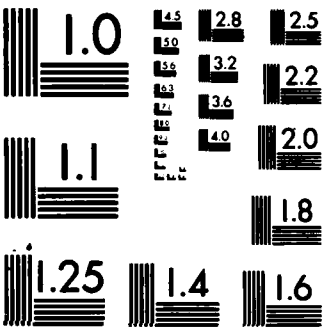

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ARACHIDONIC ACID METABOLISM BY PLATELETS OF DIFFERING SIZE

by

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The relationship between mean platelet volume (MPV) and platelet arachidonic acid metabolism was examined by studying the ability of human platelets of different size to incorporate and metabolize tritiated arachidonic acid ($(^3H)AA$). Platelet phospholipids were labelled with $(^3H)AA$ and the platelets were fractionated into size-dependent subpopulations by counterflow centrifugation. The incorporation of $(^3H)AA$ increased through the fractions proportional to the MPV. After thrombin stimulation the percent of total ^3H radioactivity released from the platelets decreased as the MPV increased.

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However, fractionation of the released ^{3}H radioactivity by HPLC (high performance liquid chromatography) demonstrated that MPV had no significant influence on the percent of total platelet ^{3}H radioactivity released as cyclo-oxygenase products or as HETE (12-hydroxyeicosatetraenoic acid) but that the release of unmetabolized (^{3}H)AA decreased as MPB increased. In separate experiments using unlabelled platelets the absolute release of thromboxane B2 (TXB_2) after collagen- and thrombin-induced aggregation was measured by radioimmunoassay and was found to increase in proportion to the MPV. These results demonstrate that the release of arachidonic acid metabolites is qualitatively similar in platelets of different size. However, the absolute ability of platelets to incorporate arachidonic acid, convert it to active metabolites and release them is proportional to their volume. The ability of platelets to release unmetabolized arachidonic acid varies inversely with their MPV.

SUMMARY

The relationship between mean platelet volume (MPV) and platelet arachidonic acid metabolism was examined by studying the ability of human platelets of different size to incorporate and metabolize tritiated arachidonic acid ([^{3}H]AA). Platelet phospholipids were labelled with [^{3}H]AA and the platelets were then fractionated into size-dependent subpopulations by counterflow centrifugation. The incorporation of [^{3}H]AA increased through the fractions proportional to the MPV. After thrombin stimulation the percent of total ^{3}H-radioactivity released from the platelets decreased as the MPV increased. However, fractionation of the released ^{3}H-radioactivity by HPLC (high performance liquid chromatography) demonstrated that MPV had no significant influence on the percent of total platelet ^{3}H-radioactivity released as cyclooxygenase products or as HETE (12-hydroxyeicosatetraenoic acid) but that the release of unmetabolized [^{3}H]AA decreased as MPV increased. In separate experiments using unlabelled platelets the absolute release of thromboxane B_2 (TXB_2) after collagen- and thrombin-induced aggregation was measured by radioimmunoassay and was found to increase in proportion to the MPV. These results demonstrate that the release of arachidonic acid metabolites is qualitatively similar in platelets of different size. However, the absolute ability of platelets to incorporate arachidonic acid, convert it to active metabolites and release them is proportional to their volume. The ability of platelets to release unmetabolized arachidonic acid varies inversely with their MPV.

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INTRODUCTION

Studies of circulating platelets have shown them to be heterogeneous with respect to volume, density, metabolism, function and age (Karpatkin, 1969; Ginsburg and Aster, 1972; Penington et al, 1976; Corash et al, 1979; Rand et al, 1981). However, there remains controversy over both the causes and relevance of platelet heterogeneity. Recent advances in electrical cell sizing techniques have made one index of platelet heterogeneity, the platelet volume distribution, routinely available in most clinical laboratories (Rowan et al, 1979; Giles, 1981; Bessman et al, 1981). However, little information is available on the activities of size-dependent subpopulations as the majority of previous studies of platelet heterogeneity have been performed on platelets fractionated on the basis of their density, which can vary independently of volume (Ginsburg and Aster, 1972; Haver and Gear, 1981). We have recently described a method for the isolation of size-dependent platelet subpopulations by counterflow centrifugation (Thompson et al, 1982a). Further, we have demonstrated that platelet response to aggregating agents such as thrombin and collagen increases in proportion to their mean platelet volume (MPV; Thompson et al, 1982b).

An important mechanism for the modulation of platelet activities such as aggregation and the release reaction is that of arachidonic acid metabolism. Platelets are capable of producing the potent aggregatory metabolite thromboxane A_2. Inhibition of this pathway leads to impaired platelet function both *in vitro* and *in vivo*. Platelets also produce prostaglandins (PGs) and the lipoxygenase derived metabolite 12-hydroxyeicosatetraenoic acid (HETE) and there is some evidence for modulation of platelet activity by these metabolites

(Marcus, 1978). To date no detailed study of arachidonic acid metabolism by platelet subpopulations has been reported. The present study was undertaken in order to define the relationship between platelet volume and arachidonic acid metabolism. Qualitatively, this was achieved by examining [^{3}H]AA incorporation and metabolism by size-dependent platelet subpopulations obtained by counterflow centrifugation. Absolute measurements were made by the radioimmunoassay of thromboxane B_2 (TXB_2) released from unlabelled platelet subpopulations.

MATERIALS AND METHODS

Blood collection and platelet isolation

Blood was obtained from healthy male laboratory personnel who had not donated blood nor taken any medications known to affect platelet function within the previous 10 days. The blood (42.5 ml) was collected via a 20-gauge needle directly into 7.5 ml acid-citrate dextrose (ACD; Formula A, Travenol Laboratories, Deerfield, IL). The ACD anticogulated blood was diluted with an equal volume of a modified phosphate buffered saline (PBS; 105.5 mM NaCl, 12.8 mM Na_2HPO_4, 2.8 mM KH_2PO_4, 1.3 mM Na_2 EDTA, 15% v/v ACD) and the platelets isolated by centrifugation at 160 x g for 7 min. Residual platelets were isolated from the remaining blood by the addition of 50 ml PBS and repeat centrifugation. This method is a slight modification of that previously described (Thompson et al, 1982a) in which PBS-A (0.5% fatty acid free BSA in PBS) was used throughout platelet isolation. Unless specified otherwise, the platelet isolation procedures described were performed at room temperature.

Platelet phospholipid labelling

Platelet phospholipids were labelled with [^{3}H]AA as follows: 300 µl of a 10% solution of delipidated BSA (Chen, 1967) containing 6.0 µCi [^{3}H]AA (78.2 Ci/mmol, New England Nuclear, Boston, MA) was added to the unfractionated platelet population and incorporation was allowed to proceed for 30 min at 37°C in a gently oscillating water bath (Rittenhouse-Simmons et al, 1976). After labelling and throughout subsequent washing and fractionation procedures, samples were collected to monitor [^{3}H]AA uptake and release.

Isolation of platelet subpopulations

Following the labelling of platelet phospholipids 10 ml of a 5% solution of BSA in PBS and 8 ml of ACD were added per 100 ml of mixture. Platelets were then sedimented at 700 x g for 15 min, resuspended in 20 ml of PBS-A, recentrifuged at 700 x g for 15 min, and taken up in 5 ml PBS-A. Isolation of seven size-dependent platelet subpopulations was performed by counterflow centrifugation as previously described (Thompson et al, 1982a). The seven fractions and a sample of the original unfractionated platelet population were placed on ice for 10 min and 100 mM EGTA (Sigma) added to a final concentration of 1 mM. The platelets were then sedimented by centrifugation at 2000 x g for 10 min, the supernatant buffer aspirated and the platelets resuspended in Tris-citrate-bicarbonate (TCB) buffer (Rittenhouse-Simmons and Deykin, 1976). In order to obtain comparable quantities of platelets in the fractions used for subsequent studies, platelets in the early and late fractions (fractions 1 and 2 and fractions 6 and 7) were pooled. The platelet count in all the final suspensions was adjusted to approximately 2.5 x 10^8/ml. BSA and $CaCl_2$ were added to a final concentration of 0.05% and 3 mM, respectively.

Platelet ^{3}H-radioactivity release

Platelet ^{3}H-radioactivity release was assessed by stirring 0.9 ml of the fractions in siliconized glass cuvettes at 37°C in a platelet aggregometer (Model 300, Chronolog, Broomall, PA). After a 2 min equilibration period platelet aggregation was initiated with 100 ul of human α-thrombin (Dr. John Fenton, New York State Department of Health) to give the final concentration required (typically 1 U/ml). After 5 min the contents of the cuvette were transferred to an Eppendorf microcentrifuge tube containing 100 µl ice-cold 10% dimethyl sulfoxide (DMSO) in 200 mM EDTA (Russell and Deykin, 1979). The sample was mixed and immediately centrifuged at 12,000 x g for 4 min in an Eppendorf Model 5412 Microcentrifuge to remove the platelets. The supernatant buffer (800 µl) was removed and a sample was counted for total ^{3}H-radioactivity release; the remainder (600 µl) was stored at -20°C for subsequent [^{3}H] AA metabolite analysis.

Released [^{3}H]AA metabolites

Metabolites of [^{3}H]AA contained in 400 µl of the above medium were measured by high performance liquid chromatography (HPLC) using a Waters High Pressure Liquid Chromatograph Model ALC-GPC244 (Waters Associates, Medford, MA) equipped with a Waters Reverse Phase Fatty Acid Column eluted with a three-phase solvent system. The method which has been described in detail previously (Russel and Deykin, 1979; Deykin et al, 1979) allows complete separation of the major platelet metabolites of arachidonic acid.

Thromboxane B_2 production by platelet subpopulations

In separate experiments unlabelled platelet subpopulations were treated with collagen (Hormon-Chemie, Munich, West Germany) or thrombin in a manner identical to that described above. The TCB-DMSO/EDTA supernates were assayed for TXB_2 by radioimmunoassay using a commercially available kit (New England Nuclear, Boston, MA). For assay the samples were diluted 100-fold or greater in TCB. In preliminary experiments, it was determined that the levels of DMSO/EDTA present did not affect the standard curve which contained an equivalent volume of TCB buffer. All assays were performed in duplicate and results calculated from the mean.

Platelet counts and sizing

Platelet counts were performed visually by phase microscopy and electronically using a Coulter ZBI Counter (Coulter Electronics, Hialeah, FL). Platelets were sized using a linear scale on a Coulter ZB counter with an H_4 Channelyzer attachment (Coulter) equipped with a 50/60 aperture. Details of these procedures have been published elsewhere (Thompson et al, 1982a).

Statistics

Statistical evaluation of data was performed by linear regression analyses (Kleinbaum and Kupper, 1978).

RESULTS

The platelet counts and the MPV of the seven platelet subpopulations are given in Fig. 1. There is a stepwise increase in the MPV through the fractions,

with the MPV of the original unfractionated platelets being found amongst the mid-range of the fractions. The distribution of the platelets in the sub-populations is also shown and corresponds to the relative frequency of similar sized platelets in unfractionated platelets.

The total incorporation of [^{3}H]AA into the original unfractionated platelet population after 30 min at 37°C was 31.9 $\pm$ 10.0% (mean $\pm$ SD, n = 8) of the added label. Following labelling, platelets were fractionated into seven size-dependent subpopulations and the individual extent of labelling of the platelets measured (Fig. 2). Absolute [^{3}H]AA incorporation increased with the fraction number in close correlation with the MPV ($p < 0.001$). However, when the individual incorporation was corrected for volume, the degree of labelling was similar for all fractions ($p > 0.1$).

The percent of total ^{3}H-radioactivity released from the platelet sub-populations after thrombin stimulation is shown in Table 1. After 5 min of stirring with 1 U/ml thrombin there was a progressive decline in the percent release of ^{3}H-radioactivity through the fractions ($p < 0.01$) and the original unfractionated platelet population gave a mid-range value.

The percent of total platelet ^{3}H-radioactivity released as cyclooxygenase products, HETE and as unmetabolized [^{3}H]AA is presented in Fig. 3. In separate experiments (results not shown) we could find no difference in the individual contribution of TXB_2, HHT (hydroxyheptadecatrienoic acid) or PGs to the total cyclooxygenase derived activity in any of the platelet subpopulations. There was no significant correlation ($p < 0.05$) between platelet size and the percent of total radioactivity released as cyclooxygenase products or as HETE. However, the release of unmetabolized [^{3}H]AA showed a significant ($p < 0.01$)

inverse correlation with the MPV. The smallest platelets released 5.2 ± 0.9% and the largest 2.4 ± 0.5% of their total radioactivity as unmetabolized [^{3}H]AA. Qualitatively similar results were obtained when platelet subpopulations were incubated with 0.5 or 2 U/ml thrombin or 10 μg/ml collagen (results not shown).

Figure 4 shows the amount of TXB_2 released from unlabelled platelet subpopulations in response to 1 U/ml thrombin and 10 μg/ml collagen. For both agents there was a stepwise increase ($p < 0.01$) in the absolute amount of TXB_2 released as the fractions go from smallest to largest. When the values are corrected for the differences in volume between the fractions, TXB_2 release is similar ($p > 0.1$) for all of the fractions including the original unfractionated population.

DISCUSSION

The incorporation of labelled arachidonic acid by size-dependent platelet subpopulations was proportional to the MPV of the platelets. The differences in incorporation of [^{3}H]AA could be entirely accounted for by the demonstrated differences in platelet volume as shown by the similarity of values when uptake was corrected for the MPV of the fractions. Since all of the platelets were labelled together prior to fractionation it is unlikely that the differential labelling was an artifact of the incubation conditions or the separation procedure. The differences may reflect one or more of the following factors associated with increasing platelet size: a greater platelet surface area to which the arachidonic acid might bind, greater amounts of those enzymes involved in the incorporation of arachidonic acid into phospholipids, or a greater pool of phospholipids (or other lipids) to accept the arachidonic acid. Previous

studies have shown that under similar incubation conditions > 98% of incorporated [^{3}H]AA is found esterified to phospholipids (Rittenhouse-Simmons and Deykin, 1981).

The relationship between MPV and total ^{3}H-radioactivity release suggests that large platelets were either less able to release arachidonic acid and its metabolites, or less responsive to thrombin stimulation as demonstrated by a progressive decline in the release of ^{3}H-radioactivity with increasing MPV. However, it is clear from our previous studies that large platelets are not less responsive to thrombin or collagen as measured by either aggregation or the release reaction (Thompson et al, 1982b). Analysis by HPLC of the distribution of the released ^{3}H-radioactivity among arachidonic acid and its metabolites indicates that the relative release of both cyclooxygenase products and HETE was similar in all fractions but that it was the reduced amount of radioactivity present in unmetabolized [^{3}H]AA that was a major factor for the total release of ^{3}H-radioactivity being less in the larger platelets. The decreased release of unmetabolized [^{3}H]AA suggests that large platelets have a greater affinity for free arachidonic acid, this is supported by the data presented in Fig. 2 which shows that the uptake of [^{3}H]AA was proportionally greater in larger platelets and suggests that a common mechanism may be responsible for the greater avidity of larger platelets for unmetabolized arachidonic acid.

Absolute measurements of arachidonic acid metabolism were made by radioimmunoassay of TXB_2 released from collagen and thrombin stimulated platelets subpopulations. We demonstrated that large platelets produced proportionally more TXB_2 than small platelets. This increased production

which reflects production of the potent aggregating agent thromboxane A_2, may contribute to the increased in vitro activity of large platelets that we have recently demonstrated (Thompson et al, 1982b). The above observations should be considered in those controlled studies in which patient or experimental platelet populations are matched by number alone, where possible in vivo and in vitro platelet mass should be considered.

These results show that the release of arachidonic acid metabolites is qualitatively similar in platelets of different size separated by counterflow centrifugation. However, the absolute ability of platelets to incorporate arachidonic acid, convert it to prostaglandins, thromboxanes and HETE, and release these metabolites, is proportional to their volume.

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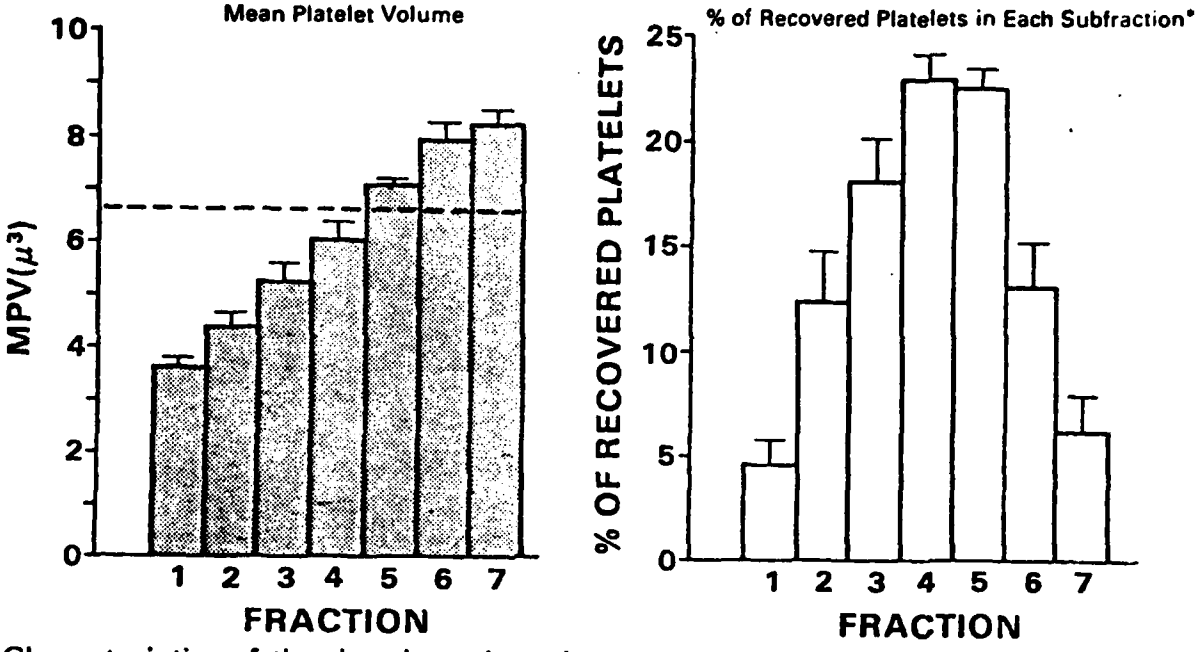
FIGURE 1



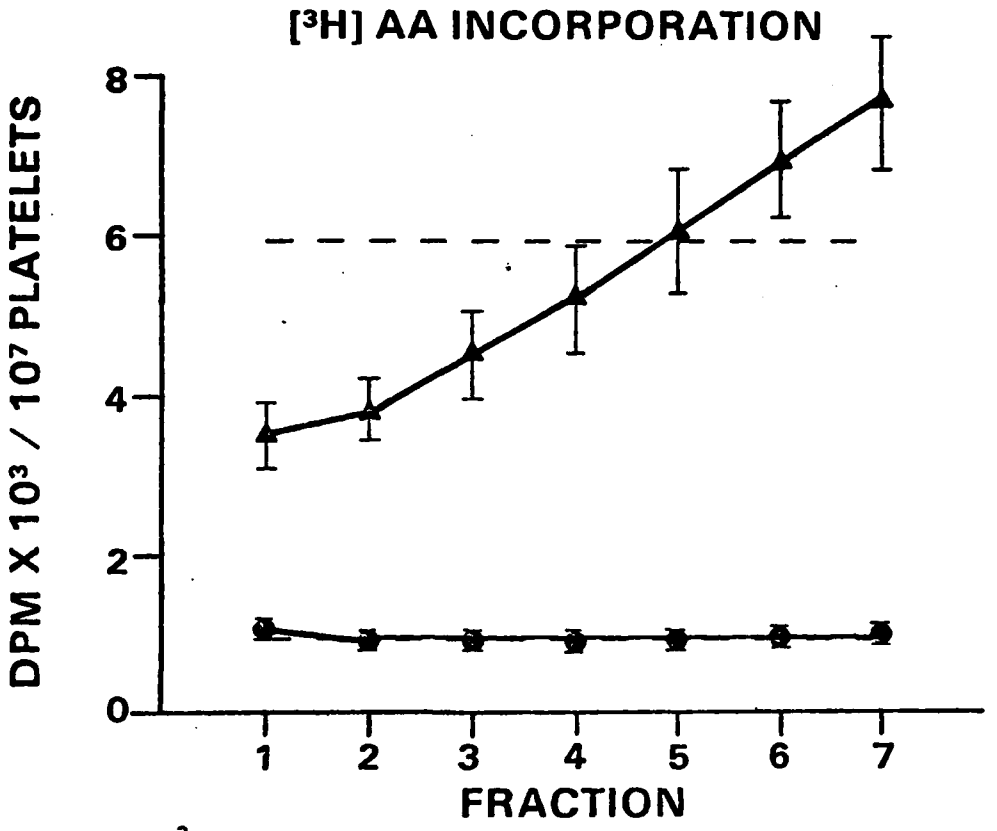
Characteristics of the size-dependent platelet subpopulations.

Results are expressed as mean ± SD, n = 8.

-----MPV of unfractionated platelets.

* Total recovery from unfractionated platelets > 90%.

FIGURE 2



The extent of [^{3}H]AA incorporation by size-dependent platelet subpopulations.

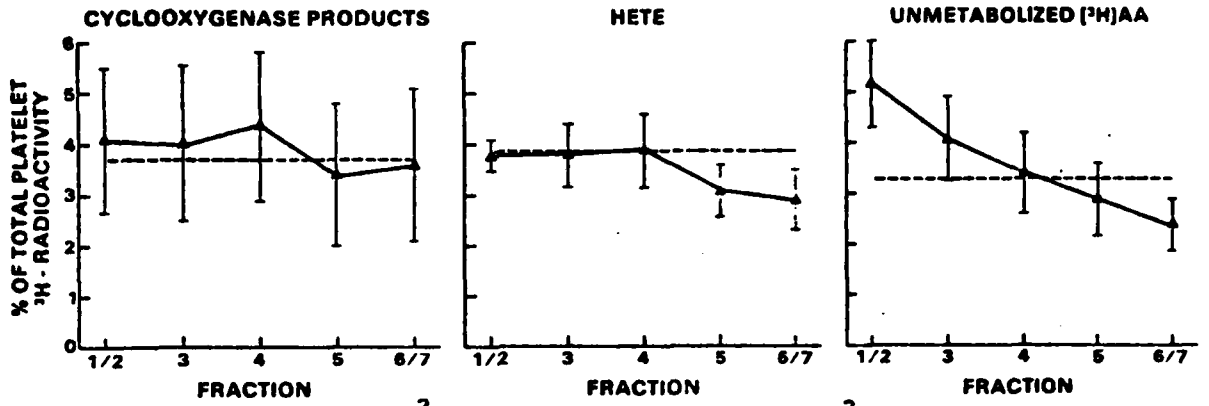
Results are expressed as mean ± SEM, n = 7.

-----Unfractionated platelets.

—▲—Absolute incorporation.

—●—Volume-corrected incorporation.

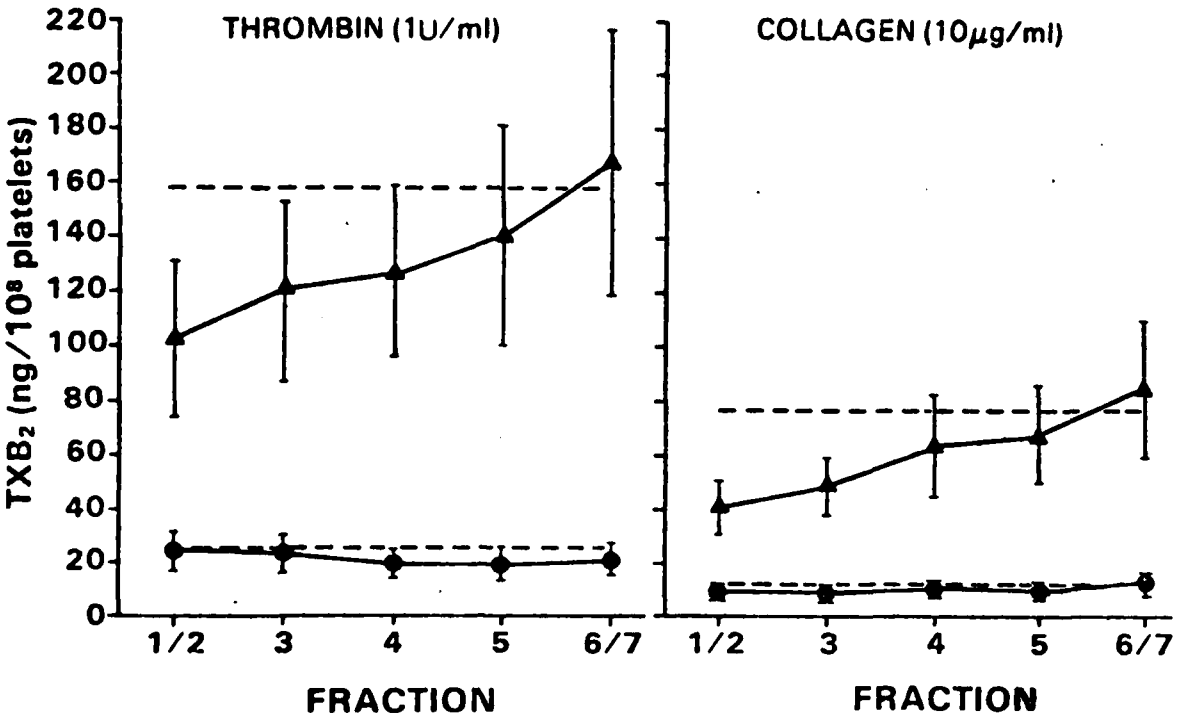
FIGURE 3



The percent of total platelet ^{3}H-radioactivity released as [^{3}H]AA and its metabolites. Incubation conditions were identical to those described in the legend to Table 1. [^{3}H]AA and its metabolites were fractionated by HPLC, cyclooxygenase products include PGs, TXB_2 and HHT. Results are expressed as mean $\pm$ SD, n=5.

-----Unfractionated platelets.

FIGURE 4



TXB_2 production by size-dependent platelet subpopulations.

Results are expressed as mean ± SEM, n = 4.

—▲—Absolute TXB_2 production.

—●—Volume-corrected TXB_2 production.

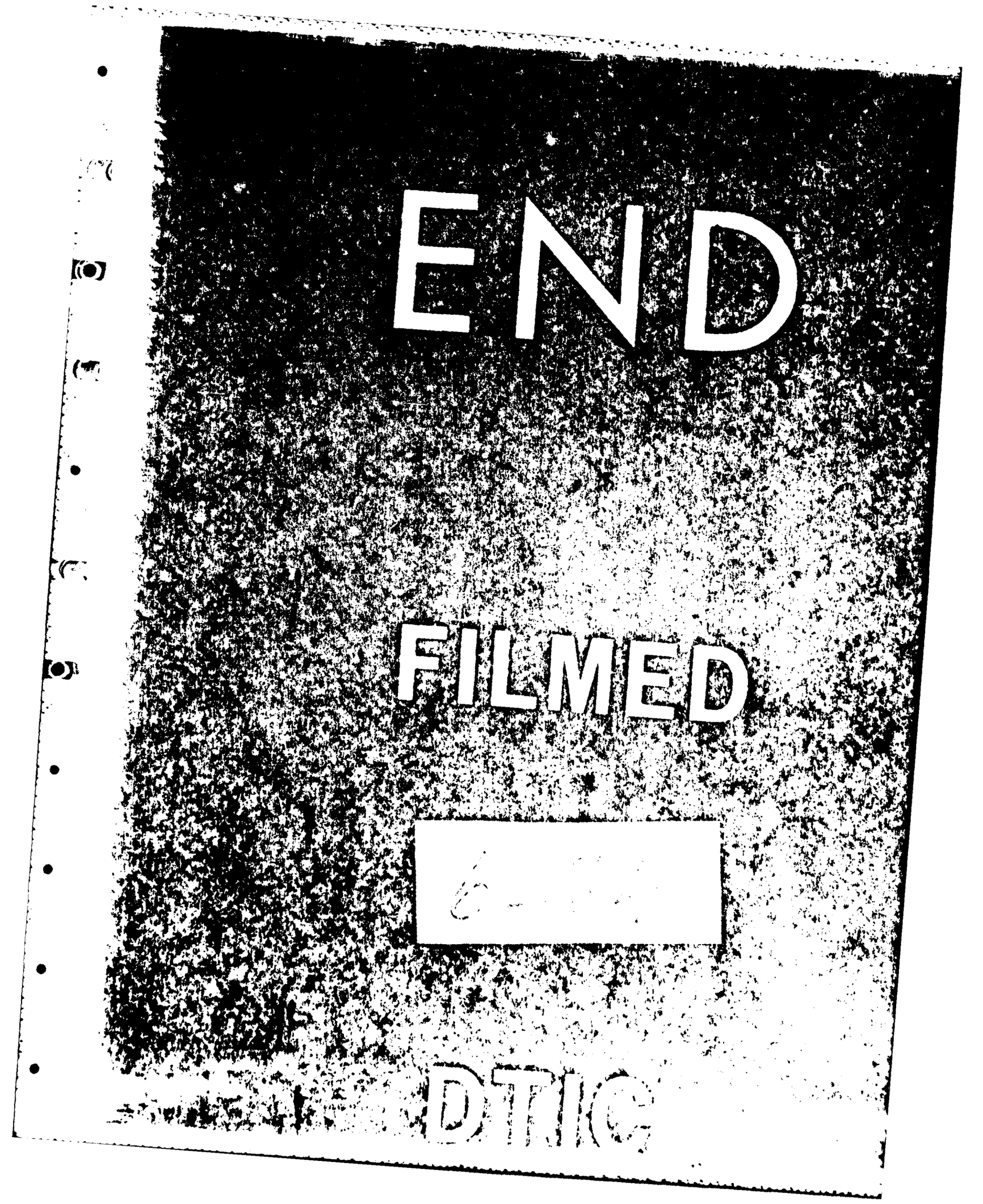
-----Unfractionated platelets.

TABLE 1

Thrombin-induced total ^{3}H-radioactivity release

Fraction	^{3}H-Radioactivity release (% total)
	Thrombin
1/2	16.4 ± 3.4
3	14.3 ± 3.0
4	13.6 ± 3.1
5	11.9 ± 2.8
6/7	11.7 ± 3.4
Unfractionated	12.3 ± 2.3

Each prelabelled platelet subpopulation (2.5×10^{8}/ml) was stimulated with 1 U/ml thrombin for 5 min at 37°C. The incubation was terminated by the addition of DMSO/EDTA and total ^{3}H-radioactivity release measured. Results are expressed as Mean ± SD, n = 5.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